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14. ABSTRACT

It is fundamentally important to understand the underlying mechanisms regulating prostatecaner (PCa) metastasis. Despite the increased PSMA expression found in more advanced stage ofPCa, little is known about the functional role of PSMA in PCa progression. Work accomplishedfor the period of the report has (1) generated the fluorescently labeled anti-PSMA antibodiesfor monitoring PSMA expressions in live PCa cells, (2) established the cell model systemswith reduced PSMA expression for studying PSMA functions, (3) identified fibronetin as aspecific extracellular matrix for enhanced LNCaP attachment and (4) performed 2-D woundhealing assays to examine the role of PSMA in PCa cell migration. Results from these studiesdemonstrated and further supported the idea that PSMA is involved in PCa cell adhesion andmigration, therefore will enhance our understanding of molecular regulatory mechanisms ofPCa. Knowledge about the action of PSMA in cell adhesion and migration during PCa metastasiswill have a direct impact on the improvement for design better-targeted approaches fortreating patients suffering from metastatic prostate cancer.

15. SUBJECT TERMS
prostate cancer, prostate-specific membrane antigen (PSMA), cell adhesion, cell motility andmigration, anti-PSMA antibody, prostate cancer metastasis

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INTRODUCTION

Metastatic prostate cancer (PCa) is a leading cause of cancer death of men in the United States. PCa metastasis is a multi-step process that involves a variety of molecules affecting the severity of the disease and the response to treatments (1-3). Prostate-specific membrane antigen (PSMA) has been recognized as the single most well-established and highly restricted membrane antigen for PCa and its expression is upregulated approximately 10-fold in advanced stages of metastasis and in hormone-refractory PCa (4-13). These observations indicate the importance of PSMA in PCa and its application in targeted clinical treatments. Previously, we demonstrated that expression of PSMA, instead of promoting cell growth and survival, results in morphologically better-spreading cells with redistributed F-actin and beta-1 integrin. Those molecules are known to play key roles in cell adhesion and motility. Furthermore, we observed motility changes in cells with their PSMA enzymatic activity inhibited by specific anti-PSMA antibody. These findings suggest a potential role of PSMA in adhesion and migration, critical processes during PCa metastasis. Currently, there is no effective treatment for advanced PCa. This proposed study is aimed to establish the role of PSMA and its enzymatic activity in PCa cell adhesion and migration which can be used to develop novel strategies for diagnosis and therapeutic treatment for human metastatic PCa.

BODY

1. Specific Aims

(1) To investigate the effect of PSMA expression on PCa cell adhesion

Anti-PSMA antibody will be conjugated directly with fluorescent probes for live cell imaging. PSMA distribution of cells grown on different extracellular matrices will be characterized to provide guidance for designing the motility assays. Assays measuring cell attachment and detachment will be employed for the identification of a functional role of PSMA in cell adhesion.

(2) To characterize the effect of PSMA expression on PCa cell motility and migration

Experiments are designed to test whether increased expression of PSMA in higher grade cancer is the result of its role in regulating cell motility and migration during PCa metastasis. Changes in cell motility and migration will be measured by *in vitro* 2D and 3D migration assays. Results from these studies will directly indicate the function of PSMA in the particular stages during PCa progression.

(3) To determine whether the enzymatic activity of PSMA directly regulates the adhesion and migration of PCa cells.

Cell attachment, detachment and migration assays will be performed in the presence or absence of J415, a specific anti-PSMA antibody with measurable inhibitory effect on PSMA enzymatic activity. In addition, a possible link between PSMA enzymatic activity and integrin activity and function will be examined using J415 and P4C10, a functional inhibitory anti-integrin antibody. Demonstrating a connection between PSMA enzymatic activity and integrin activity will advance our understanding of the regulatory machinery controlling PCa metastasis.

2. Study and Results

Despite the consistent finding of the correlation of upregulated PSMA with increased PCa aggressiveness, the functional role of PSMA in PCa metastasis is unknown. Previously, we have found that expression of PSMA results in morphologically better-spreading PCa cells with redistributed F-actin and increased integrin levels. We also found an increased PCa cell motility in cells with their PSMA enzymatic activity inhibited by a specific anti-PSMA antibody. These findings suggest a potential role of PSMA in PCa adhesion and migration which are critical processes during metastasis. This proposed study is aimed to establish the role of PSMA in PCa cell adhesion and migration. For the first funding year, we have successfully accomplished the goals and studies proposed in the task 1 of the approved Statement of Work. In the following, we describe in details of our research findings and provide a complete record of our accomplishments for the period of this report.

Task 1. To investigate the effect of PSMA expression on PCa cell adhesion (Months 1-12)

1a. Generate fluorescent-conjugated antibodies for live cell adhesion and motility assays

Rationale and Research Accomplishment:

Conjugating fluorescent dyes directly to an antibody has been demonstrated to be a powerful tool for investigating protein functions (14-15). Accurately measuring PSMA expression levels in live cells is essential when assessing the role of PSMA. We have successfully generated several photo-stable fluorescent probes Alexa488, Alexa546 and Alexa633-conjugated anti-PSMA antibodies (J591 and J415). We have examined the binding characteristics of these antibodies after the conjugation and found that conjugating the antibody with fluorescent probes did not alter their binding properties. These fluorescent-conjugated antibodies have been used to monitor PSMA expression and for the examination of its functional role in cell adhesion (see below). They will also be used for future studies (proposed in task 2-3) to establish the role of PSMA in cell migration and its association with integrin in live PCa cells.

Methods:

Monoclonal antibodies (mAbs) J591 and J415 directly against extracellular epitopes of PSMA have previously been described (16). Photo-stable fluorescent probes, Alexa488, Alexa546 and Alexa633 (Molecular Probes) were conjugated separately to anti-PSMA antibodies (J591 and J415) following the manufactory's instruction. Briefly, unlabeled antibodies were dissolved in pH=9 sodium bicarbonate solution before mixing with the dye in dark for 1 hour at room temperature. PD-10 sizing column was used to separate the free dye and large aggregates from the conjugated antibodies. The properties of the conjugated-antibodies were evaluated in the presence of excess amount (50x) of un-labeled antibodies before application to live cells.

PC3 cells (PSMA-negative, CRL-1435), LNCaP cells (PSMA-positive, CRL-1740) and 22Rv1 cells (PSMA-positive, CRL-2505) were obtained from ATCC. PC3-PSMA cells are PC3 cells stably expressing PSMA (17). All the cells were grown in RPMI medium containing 10% FBS,

100 units/ml penicillin-streptomycin. The cells were grown at 37°C in a humidified atmosphere of 5% CO_2.

For microscopy, cells were grown to subconfluence on poly-D-lysine treated coverslips For incubations of live cells with antibodies, cells were treated as described previously (18). Briefly, cells were incubated with fluorescence-conjugated antibodies in a 37°C incubator under an atmosphere of 5% CO_2. After incubation, cells were washed and subjected to live cell imaging or fixation. Fixation was performed with 3.3% paraformaldehyde in PBS for 15 min at room temperature. For indirect immunofluorescence labeling, fixed cells were permeabilized with 0.025% (wt/vol) saponin (Calbiochem, San Diego, CA) in PBS.

Results, Significance, Obstacles and Alternative Approaches:

We have generated several different fluorescent dye conjugated anti-PSMA antibodies J591 and J415. PSMA negative PC3 cells, PC3 cells stably transfected with PSMA (PC3-PSMA), PSMA-positive LNCaP cells and 22Rv1 cells were used to examine the binding properties of these conjugated antibody. After binding, cells were rinsed with PBS three times and live cell images were immediately collected to view the fluorescent labeling. Specific labeling of anti-PSMA antibody was obtained in LNCaP cells (Fig. 3A in the Appendices and Supporting Data section), in 22Rv1 cells (Fig. 6A-C in the Appendices and Supporting Data section) and in PSMA-positive PC3-PSMA but was not observed in PSMA-negative PC3 cells (data not shown). The binding of fluorescently labeled anti-PSMA antibody was completely blocked by incubating cells with excess amount of unlabeled antibody. These fluorescent-conjugated antibodies have been and will continuously be used to monitor PSMA expression and examine its functional relationship in cell adhesion (Aim. 1), migration (Aim. 2) and with integrin (Aim. 3) in live cells.

Conjugating fluorescence to functional blocking antibody is complicated since its functional blocking ability can potentially be affected during the conjugation. To fluorescently label the functional blocking anti-integrin antibody, we evaluated the anti-α5 integrin antibody P1D6 (Chemicon) shown to be suitable for attachment inhibition. As an unlabeled antibody, it bound to live LNCaP cells well. Once we can confirm its ability to functionally block LNCaP cell attachment, we plan to use it to examine the relationship between PSMA and integrin. We also plan to test functional blocking anti-β-1 integrin antibody P4C10 (Life technologies). We will conjugate the fluorescent probes to these functional blocking antibodies for live cell experiments.

1b. Optimize PSMA siRNA transfection in LNCaP cells to reduce PSMA expression

Rationale and Research Accomplishment:

One of the most important accomplishments during this first year of funding is that we were able to significantly increase the efficiency of transfection with siRNA of PSMA. We established a protocol that, with transient transfection of siRNA of PSMA, we could achieve an approximately 90% reduction of PSMA expression in LNCaP (shown in Fig. 1A-C in Appendices and Supporting Data section). Furthermore, we were able to further develop a conditional medium to allow LNCaP cell to maintain the reduced PSMA expression for up to 2-weeks. Usually, the effect of transient transfection peaks at 3-5 days after transfection before losing its targeted effect. Achieving a significant reduction of PSMA expression and especially maintaining PSMA

expression at a reduced level (see in Fig. 1A-B in Appendices and Supporting Data section) will enable us to perform the critical functional studies that otherwise won't be possible.

Methods:

To achieve a high level of transfection efficiency, four different siRNAs of PSMA obtained from Dharmacon were transfected either individually or as a pool to LNCaP cells. LNCaP cells were incubated with a mixture of different concentrations (2-10 ul) of undiluted Dharmafect3 and 10-100 nM siRNA-PSMA for 5 hours. Cells were then incubated in regular growth medium for three days or were switched to growth medium containing 1/3 of the transfection mixture for indicated period of times (Fig. 1A-B). Cell lysate was collected after siRNA transfection for 3 days, 1 week and 2 weeks and Western blot analysis was performed to evaluate the extent of PSMA reduction. Live cells labeled with fluorescent-J591 were used for FACS analysis.

Results, Significance, Obstacles and Alternative Approaches:

Our preliminary data showed that it is possible to decrease PSMA levels in LNCaP cells with transfection of siRNA against PSMA. However the success rate is low. Enhanced siRNA transfection efficiency would greatly help to achieve the goals in this proposed work. For the report period, we have focused our effort to establish a siRNA transfection protocol that would reduce the PSMA expression significantly in LNCaP cells by testing and systematically evaluating the various factors involved in siRNA transfection, including the concentration of lipid carrier, the amount of siRNA, the medium of choice and the number of cells plated for transfection. The levels of PSMA expression (or reduced expression) were assessed using unlabeled or fluorescently conjugated J591 (described above in 1a).

Cell lysate from untransfected LNCaP cells were used as control for the detection and measurement of the normal PSMA expression in LNCaP cells (Fig. 1A-B). In (Fig. 1B), PSMA expression levels detected on the Western blot (Fig. 1A) were quantified and plotted to demonstrate the extent of PSMA reduction. As shown in Fig. 1A-B, a significant reduction of PSMA (about 90%) was observed after 1-2 weeks of transfection with siRNA. To further monitor the siRNA transfection efficiency and to access the specificity of siRNA-PSMA, we conducted FACS analysis on live LNCaP cells transfected with 10nM of siRNA-PSMA mixed with different amount of Dharmafect3 (2-10ul) (see Fig. 1C in the Appendices and Supporting Data section). A reduction of PSMA expression at 89.1% was observed.

To study the function of PSMA, it is important to achieve significant reduction of PSMA in LNCaP cells, particularly to maintain cells at a reduced PSMA expression level for prolonged period of time. We found that although various factors were critical for the improvement of siRNA transfection, the type of lipid carrier used for the transfection made the biggest difference for the increased transfection rate. When Dharmafect3 (Dharmacon) were used for transfection, we were able to reach the greatest improvement for the inhibition of PSMA expression in LNCaP cells (90% reduction in PSMA expression shown in Figure 1 in the Appendices and Supporting Data section). We were able to further modify the cell growth condition after the transfection so that the reduction of PSMA expression lasted for two weeks. Such outcome can be tremendously helpful to assess the function of PSMA in adhesion and motility as shown Fig. 5 and as proposed in Aim 2 and Aim 3.

Currently, we are able to get LNCaP cells maintained at a reduced PSMA level for up to two weeks. This is a major accomplishment given the fact that LNCaP cells are noted to be extremely sensitive to transfection conditions and cell detachment and death occurred frequently under culture condition that is required for transfection. We will continue to work on improving the transfection efficiency to further decrease PSMA expression and prolong the siRNA effect.

1c. Examine the characteristics of PCa cells with varied PSMA expressions grown on different extracellular matrices

Rationale and Research Accomplishment:

Cell adhesion is greatly affected and in partly controlled by the extracellular environment. Previous studies have shown that the polarized distribution of adhesion molecules is substrate-dependent (19). We have observed a dramatic increase in integrin expression in PC3-PSMA cells when compared with PC3 cells. To examine whether PSMA play a role in cell attachment and motility, during the period of report, we tested cell adhesion property of LNCaP cells and PC3 cells on dishes coated with a range of different substrate materials including: uncoated regular glass, coverslips coated with poly-D-lysine, BSA, fibronectin (FN) and collagen. PCa cell adhesion properties were characterized by first examining the morphology of cells and the number of cells plated onto various matrix materials. Cell spreading assay (20) was an approach previously used to examine the extent of cell adhesion. We found that LNCaP attachment was greatly enhanced in dishes coated with FN. It is evident that, shortly after plating, most of the LNCaP cells were only firmly attached onto the FN-coated dishes with well-spread morphology but not onto other extracellular matrices coated dishes. No significant differences in cell adhesion were observed in PC3 cells plated on dishes coated with any of the above extracellular matrices. Our findings suggest a potential interaction between PSMA and integrin, since it is known that fibronectin binds to integrin to promote various cellular functions including cancer progression.

Methods:

Cell attachment assay were performed as described before (21-22). Both PSMA-positive LNCaP cells and PSMA-negative PC3 cells were plated on uncoated dishes or dishes previously coated with 2% BSA, 10ug/ml extracellular matrix (ECM) or 10ug/ml fibronectin (FN). Cells grown on different matrix molecules-coated 96 well dishes were seeded at a density of 10,000/cm^2 (2800 cells/150 μl), containing RPMI medium without serum. The 96-well dishes were incubated at 37°C at 5% CO_2 for a maximum of 3 hours with 15 min interval before analysis. After testing many different time intervals, we found that 60 min plating was the best condition, especially when washes were involved after plating the cells. For each experiment, three random fields were imaged and counted. Non-spreading cells were defined as round phase-bright cells and well-spreading cells were defined as those had extended processes, lacked a round morphology and not phase bright.

To test cell adhesion in relationship to PSMA expressions, we modified the cell attachment assay described above by measuring the number of cells attached before and after washes. Each well (of triplicates) was washed three times with serum-free medium to remove unattached cells and

attached cells will be fixed for 20 min with paraformaldehyde at room temperature. Cell number was counted by averaging four non-overlapping microscopic fields.

Results, Significance, Obstacles and Alternative Approaches:

To examine whether PSMA play a role in cell attachment and motility, during the period of report, we tested cell adhesion property of PSMA positive LNCaP cells and PSMA negative PC3 cells on dished coated with a range of different substrate materials.

We found morphologically better spreading LNCaP cells when plated onto FN-coated dishes (shown in Fig. 2 A-H in the Appendices and Supporting Data section) but not uncoated dishes or dishes coated with either BSA or ECM (Fig. 2A-C and 2E-G). When these dishes were washed to eliminate unattached or loosely attached cells, significant reduction in cell numbers was observed of LNCaP cells plated on to uncoated dishes or dishes coated with BSA or ECM. On the contrary, not only better spreading LNCaP cells were observed when plated on FN-coated dishes (Fig. 2D and H), enhanced attachment and higher number of cells were see when LNCaP cells plated on FN-coated dishes (Fig. 2H) when compared with cells plated on uncoated dishes or dishes coated with either BSA or ECM (Fig. 2A-C and 2E-G). Furthermore, no significant differences were observed in cell morphology, attachment or cell number was observed of PC3 cells plated on any of the above matrix materials. We noted that the PC3 cells plated on to uncoated dishes, after washes, displayed a more rounded morphology. Further studies will be conducted to examine this phenomenon.

Characterizing the PSMA-positive cell adhesion, especially identifying fibronectin as a specific matrix to enhance the adhesion of LNCaP cells further suggest the role of PSMA in cell attachment and motility. These results, along with our previous work indicate the potential interaction of PSMA with integrin, a molecule known to be involved in cell adhesion and cell signally pathways to promote tumor metastasis. Moreover, these results will be helpful to select the optimal condition for the adhesion and motility assays proposed in this study (Aim 1d and Aims 2-3).

We have encountered some technical difficulties while conducting cell adhesion experiments. We found that in 96 well dishes, often after plating, significant number of cells were attached at the side of the 96 wells rather than evenly distributed in the well. This is problematic for accurately counting cell numbers. To overcome this, we modified the plating procedure to leave the cells at room temperature for 5 min before switching to the 37°C incubator. This seemed to work well and we were able to get cells evenly plated onto the dishes. Collagen coating appeared to be a challenge, although we have been able to collect data from cells plated on collagen-coated dishes. We are still working on experimental details to achieve a consistent, even and thin layer of collagen coating on the dishes for conducting adhesion and motility assays.

1d. Perform live cell assays to characterize the relationship between PSMA expression and cell attachment and detachment

Rationale and Research Accomplishment:

Previously, we have observed a dramatic alteration in cell morphology, F-actin filament distribution and integrin expression in PC3-PSMA cells when compared with PC3 cells. To examine whether PSMA expression directly regulate cell adhesion, therefore to modulate PCa migration, using *in vitro* cell model systems and live-cell imaging methods, we characterized the role of PSMA in cell motility and adhesion. We demonstrated, using wound-healing assay that PSMA expression reduced cell motility. After wounding, significant numbers of PSMA-negative PC3 cells migrating into the wounded area could be observed. In contrast, limited migration was observed in PC3-PSMA (see Fig. 3 in the Appendices and Supporting Data section). We also monitored and recorded live cells motility of PC3 and PC3-PSMA (see Fig. 4 A-B in the Appendices and Supporting Data section). When live cell migration was traced and measured, we observed a significant decrease in the distance of cell migration in PC3 cells expressing PSMA (see Fig. 4C in the Appendices and Supporting Data section). Finally, we tried to detect any alteration in cell motility in LNCaP cells with reduced PSMA expression by siRNA-PSMA. We found that reduction of PSMA expression in LNCaP cells increase cell motility (Fig. 5). Together, these observations provide evidence fors the role of PSMA in PCa cell migration.

Methods:

Fluorescent-conjugated anti-PSMA antibodies (obtained from 1a) were used to monitor the expression of PSMA. Wide field fluorescence microscopy was performed on a DMIRB inverted microscope (Leica Inc., Deerfield, IL.). Confocal images were collected on an LSM510 laser scanning confocal unit (Carl Zeiss Inc.) attached to an Axiovert 100M inverted microscope (Zeiss). The excitation on the Leica microscope was by a Hg arc 100W lamp with standard optics. Excitation on the LSM510 confocal microscope was with 25-mW Argon laser emitting at 488nm or a 1.0-mW helium/neon laser emitting at 543nm. Emissions were collected using a 505-530-nm band pass filter to collect Alexa488 and a 560-615-nm band pass filter to collect Cy3 and Alexa546 emission. Images were processed using MetaMorph image-processing software (Universal Imaging, Molecular Devices, Sunnyvale, CA). LNCaP siRNA-PSMA transfection was carried out as described in 1b. To label F-actin filaments in cells, Alexa-488 conjugated phalloidin was incubated with cells after fixation and permeabilization (described in 1a).

Results, Significance, Obstacles and Alternative Approaches:

To test the relationship between PSMA expression and cell attachment, using *in vitro* cell model systems and live-cell imaging methods, we characterized the role of PSMA in cell motility and migration. We demonstrated, using wound-healing assay that PSMA expression reduces cell motility when compared with control PSMA-negative PCa cells. Six hours after wounding, significant numbers of PSMA-negative cells migrating into the wounded area could be observed (Fig 3 in the Appendices and Supporting Data section). In contrast, limited migration was observed in PC3 cells expressing PSMA (see Fig. 3 in the Appendices and Supporting Data section). We also recorded cell motility of PC3 and PC3-PSMA (see Fig. 4 A-B in the Appendices and Supporting Data section). When live cell migration of these cells was measured (see Fig. 4C in the Appendices and Supporting Data section), a significant decrease in the distance of cell migration was observed in PC3 cells expression PSMA. As matter of fact, there is very limited movement in PC3-PSMA cells. Finally, we tried to detect any alteration in cell motility in LNCaP cells with reduced PSMA expression by transfecting siRNA of PSMA. We found that reduction of PSMA expression in LNCaP cells increases its motility when compared

with control LNCaP cells (see Fig. 5 in the Appendices and Supporting Data section). These results are consistent with our previous findings that increased cell migration was observed in cells with reduced PSMA enzymatic activities.

Moreover, we have tested the expression of PSMA in 22Rv1 cells, a PSMA-positive PCa cell line. Interestingly, we found three different levels of PSMA expressions in 22Rv1 cells and most strikingly, redistribution of F-actin was observed in 22Rv1 cells expressing high level of PSMA (see Fig. 6 in the Appendices and Supporting Data section). This result is inline with our previous finding of altered F-actin in PC3-PSMA cells. These data indicate the possibility of establishing a cell model system, without transfection, for the study of PSMA. In light of these findings, we plan continue to work with 22Rv-1 cells in addition to PC3 and LNCaP cells for the characterization and understand the role of PMSA in PCa progression.

Together, all these observations provide further evidence to demonstrate the role of PSMA in PCa cell motility. A correlated change in cell motility and PSMA expression further suggest a functional role of PSMA in promoting cell attachment.

Thus far, cell motility and migration assays were conducted on dishes collated with poly-D-lysine. We noticed that PC3 cells migrated relatively fast on these dishes and a drastic change can be observed when these fast moving cells were transfected with PSMA (shown in Fig. 5). LNCaP cells do not move significantly under our experimental conditions and it takes a relatively long time (hours to days) to observe significant changes in their motility. To overcome this, we plan to test the rate of LNCaP cell migration on FN-coated dishes and other extracellular matrices-coated dishes (data from 1c), In addition, as mentioned above (Result section in 1c), we are going to test cell attachment on dishes coated with thin layers of collagen. With improved motility assay for LNCaP cells, we can better evaluate PSMA function in cell adhesion and migration.

KEY RESEARCH ACCOMPLISHMENTS:

1. We have generated fluorescently conjugated anti-PSMA antibodies that can be used for live cell imaging.

2. We have optimized the siRNA-PSMA transfection and achieve an approximately 90% reduction of PSMA expression in LNCaP cells. This will allow us to examine the role of PSMA in PCa cells that normally expresses PSMA.

3. We were able to further modify the protocol described above (#2) by developing a conditional medium to allow LNCaP cell to maintain the reduced PSMA expression for up to 2-weeks. Achieving a significant reduction of PSMA expression and especially maintaining PSMA expression at a reduced level will be tremendously helpful to assess the functional role of PSMA.

4. We tested cell adhesion property of PSMA-positive LNCaP cells and PSMA-negative PC3 cells with a range of different matrices including: uncoated regular glass, coverslips coated with poly-D-lysine, BSA, fibronectin and collagen. We found that LNCaP attachment was greatly enhanced with fibronectin coated on the dishes.

5. No significant differences in cell morphology, attachment and cell number were observed of PSMA-negative PC3 cells plated on any of the matrix materials (#4) tested.

6. We found fibronectin specifically enhance LNCaP cell adhesion, since significant higher numbers and well spread LNCaP cells stayed attached to fibronectin-coated dishes the dishes after washes, while very limited and rounded LNCaP cells stayed attached to dished coated with other matrix materials.

7. Expression of PSMA in PSMA negative PC3 cells reduces cell motility remarkably.

8. We have tested and established the cell model system using LNCaP cells transfected with siRNA of PSMA. We found that increased LNCaP cell motility is associated with reduced PSMA expressions

9. Using fluorescently conjugated anti-PSMA antibody (generated in 1), we have found three different levels of PSMA expressions in 22Rv1 cells.

10. Redistribution of F-actin was observed in 22Rv1 cells expressing high level of PSMA.

REPORTABLE OUTCOMES:

Two manuscripts are currently in preparation for publication:

1. Lin SX, Navarro V, Liu H, and Bander, NH. A functional role of PSMA in prostate cancer cell migration. Manuscript in preparation.

2. Lin SX, Navarro V, Liu H, and Bander, NH. PSMA is involved in cell adhesion by interacting with integrin in prostate cancer cells. Manuscript in preparation.

Presentation and Abstract:

1. Lin, SX, Navarro V, Liu H and Bander NH. A Functional Role of Prostate-Specific Membrane Antigen in Prostate Cancer Metastasis. AACR meeting at Washington DC, 2006.

CONCLUSION

It is fundamentally important to understand the underlying mechanisms regulating PCa progression. Emerging clinical and research work reveals upregulated PSMA with increased PCa aggressiveness of cancer (9-10). Recently, it was shown that PSMA was associated with actin binding protein filamen A and it was reported that suppression of PSMA expression increases invasiveness, and expression of inactivating PSMA mutants reduces invasiveness (23-24). Up to date, despite the increased PSMA expression found in more advanced stage of PCa, little is known about the functional role of PSMA in PCa progression. The outcome of the proposed studies will provide direct evidence on whether upregulated PSMA expression in later stage and increased PCa aggressiveness is the result of its role in cell attachment, adhesion and migration during PCa progression.

Work accomplished for the period of the report has (1) generated the fluorescently labeled anti-PSMA antibodies for monitoring PSMA expressions in live PCa cells, (2) established the ideal cell model systems with reduced PSMA expression for functional studies, (3) identified fibronetin as a specific extracellular matrix for enhanced LNCaP attachment and (4) performed 2-D wound healing assays to examine the role of PSMA in PCa cell migration. Results from these studies demonstrated and further supported the idea that PSMA is involved in PCa cell adhesion and migration, therefore will enhance our understanding of molecular regulatory mechanisms of PCa. Work from the period of the report also generated the critical tools (agents and model systems) for our continuing efforts (proposed in the study) for the understanding of PSMA function in prostate cancer progression.

In the following funding years, we will continue to pursue the aims as outlined in the approved Statement of Work. We plan to establish the cell model system using siRNA of PSMA that we have generated to modify the expression of PSMA. Once such a model system can be established, we will characterize the specific step and role of PSMA in cell motility using various approaches including 2D and 3D and wounding hearing assays as proposed in Aim 2. We will evaluate the ability of cell migration on different matrixes from the data we have obtained as mentioned above. We will generate fluorescent conjugated anti-integrin antibody to test whether PSMA and integrin interact directly as proposed in Aim 3, using live cell microscopy. Finally, J415, the specific anti-PSMA antibody with measurable inhibitory effect on PSMA enzymatic activity and functional inhibitory anti-integrin antibodies will be used to determine the role of PSMA in cell attachment, detachment and migration. Results from these studies will help us to understand the interaction of PSMA expression and enzymatic activity with integrin activity, therefore it will enhance our understanding of molecular regulatory mechanisms of PCa.

The outcome from will study will have a direct impact on the improvement of treatments including immunotherapy for human PCa. Knowledge about the action of PSMA in specific event during PCa metastasis will help to design better-targeted approaches for treating patients suffering from metastatic prostate cancer. Currently, monoclonal antibodies against PSMA tested in Phase I-II clinical trials have shown great potential. Identifying the functional role of PSMA in cell adhesion-related events will help to provide guidance for choosing the best suited antibody and the most appropriate toxins for conjugation, therefore to maximize the therapeutic efficacy and reduce side effects for human PCa immunotherapeutic treatment.

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25. APPENDICES and SUPPORTING DATA:

Total of 6 figures are included and attached in this report.

Figure 1. Reduction of PSMA Expression by Transfection with siRNA-PSMA

Figure 2. Attachment of PCa cells on Various Extracellular Matrices

Figure 3. Effect of PSMA Expression on PSMA-Negative PC3 cell Migration

Figure 4. Cell Motility of PC3 and PC3-PSMA Cells

Figure 5. Effect of Reduced PSMA Expression on LNCaP Cell Motility

Figure 6. Distribution of F-actin in 22Rv1 Cells with Different PSMA Expression Levels

Figure 1. Reduction of PSMA Expression by Transfection with siRNA-PSMA

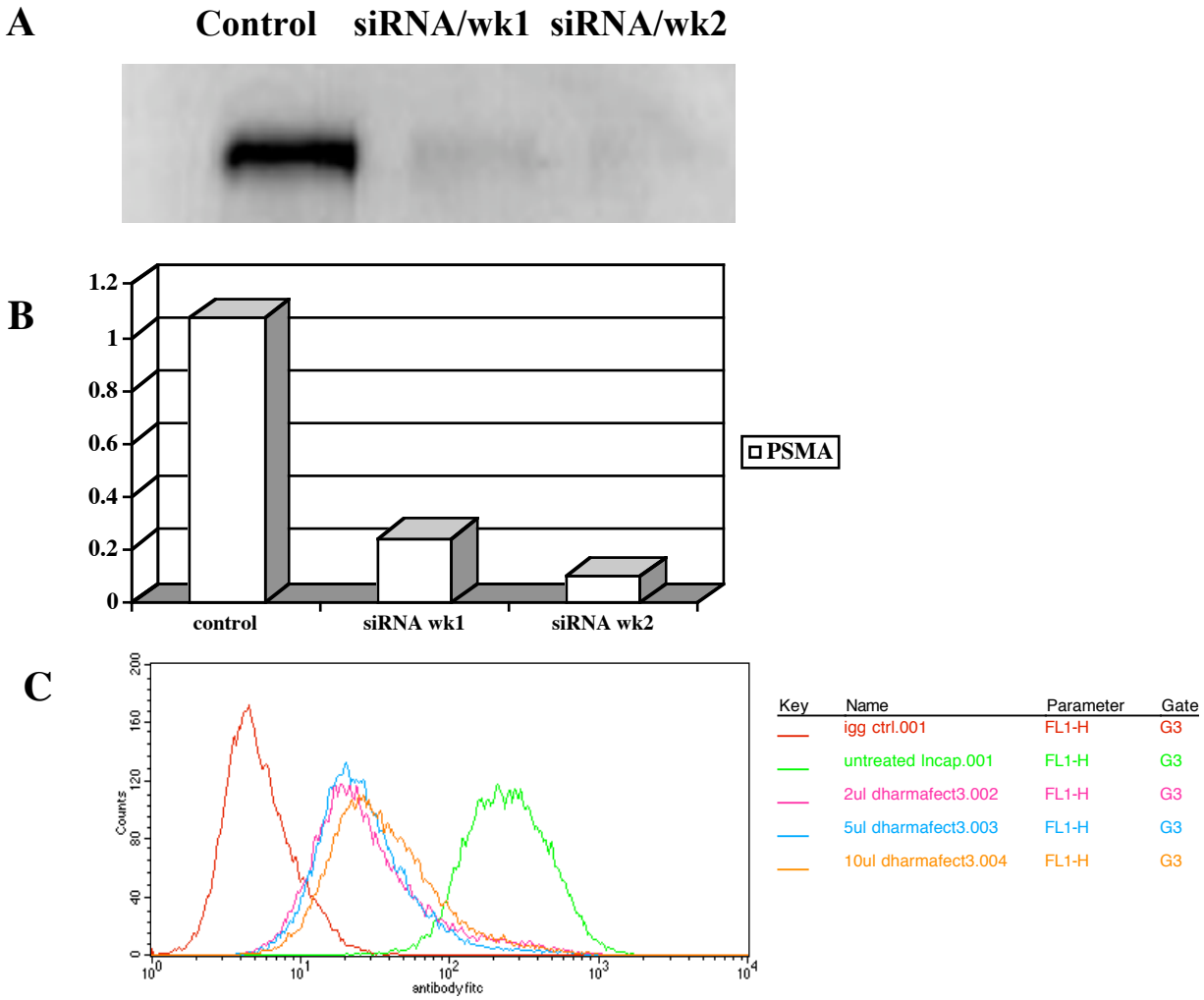

Figure 1. Transfecting siRNA of PSMA to LNCaP cells reduces their PSMA expression. LNCaP cells were transfected with siRNA of PSMA by incubation with a mixture of siRNA and Dharmafect3 for 5 hrs before switching to growth medium containing 1/3 of the transfection mixture for indicated period of times. (A), Cell lysate was collected after siRNA transfection for 1 week or 2 weeks and Western blot analysis was performed to evaluate the extend of PSMA reduction. Cell lysate from untransfected LNCaP cells were used as control for normal PSMA levels in LNCaP cells. (B), the protein levels in A were measured, quantified (The level of PSMA expression was normalized with the actin) and plotted to demonstrate the PSMA levels shown in A. (C), FACS analysis was conducted in cells transfected for 3 days with different amount of Dharmafect3 (2-10ul) and the level of PSMA expression on cells were plotted.

Result: Reduction of PSMA, approximately 90%, was observed in LNCaP cells transfected with siRNA of PSMA. This reduction of PSMA expression lasted for prolonged period of time. Significant reduction of PSMA expression was achieve using a range of Dharmafect3 at 2-10ul/ml mixed with 20nM siRNA-PSMA for transfection.

Figure 2. Attachment of PCa cells on Various Extracellular Matrices

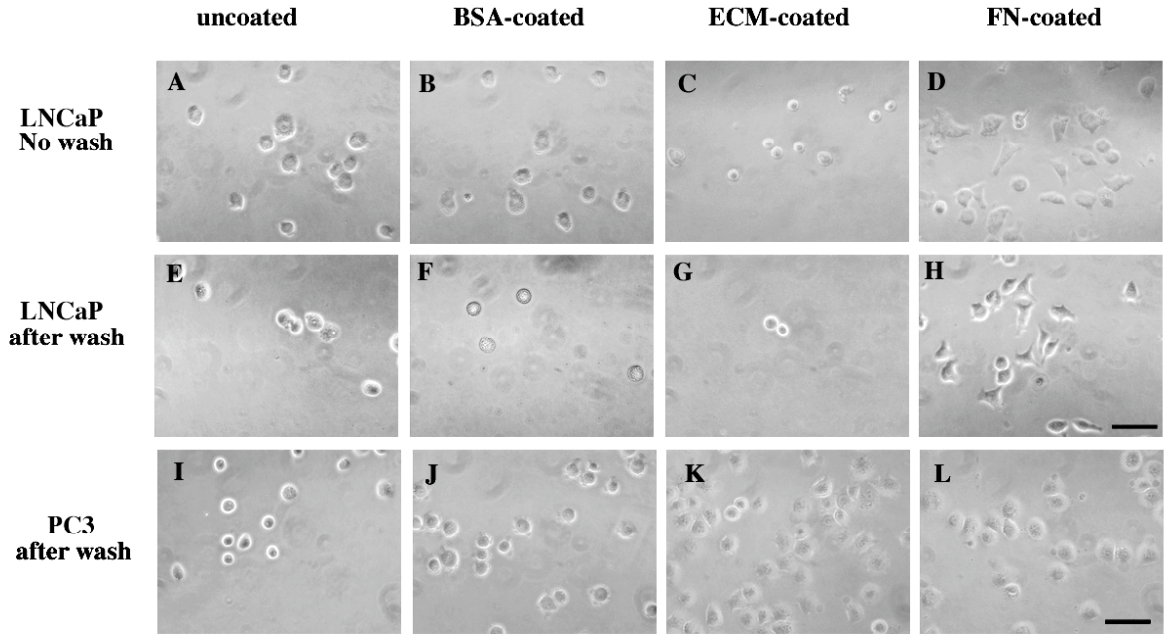

Figure 2. Increased LNCaP adhesion was observed in dishes coated with fibronectin. PSMA-positive LNCaP cells (A-H) and PSMA-negative PC3 cells (I-L) were plated onto triplicates of uncoated dishes (A, E, and I) or dishes coated with 2% BSA (B, F, and J), 10ug/ml extracellular matrix (ECM) (C, G and K) or 10ug/ml fibronectin (FN) (D, H and L). Cells were plated for 60 min without wash (A-D) or washed with serum free RPMI medium for 3 times (E-L). After washes, all the unattached cells were washed away, leaving only the adherent cells for imaging collection. Bar=20um.

Results: Morphologically better spreading PSMA-positive LNCaP cells were onto FN-coated dishes (D) but not of cells plated on uncoated dishes or dishes coated with either BSA or ECM (A-C, respectively). Enhanced cell attachment and higher number of cells were observed in LNCaP cells plated on Fn-coated dishes after washes (H). Reduced numbers of LNCaP cells were left on uncoated dishes after wash (E). Very limited cells could be seen on dishes coated with either BSA or ECM (E-G, respectively). On the other hand, no significant differences observed in cell morphology, attachment and cell number were observed of PSMA-negative PC3 cells plated on any of the matrix materials tested (I-L) with or without washes.

Figure 3. Effect of PSMA Expression on PSMA-Negative PC3 cell Migration

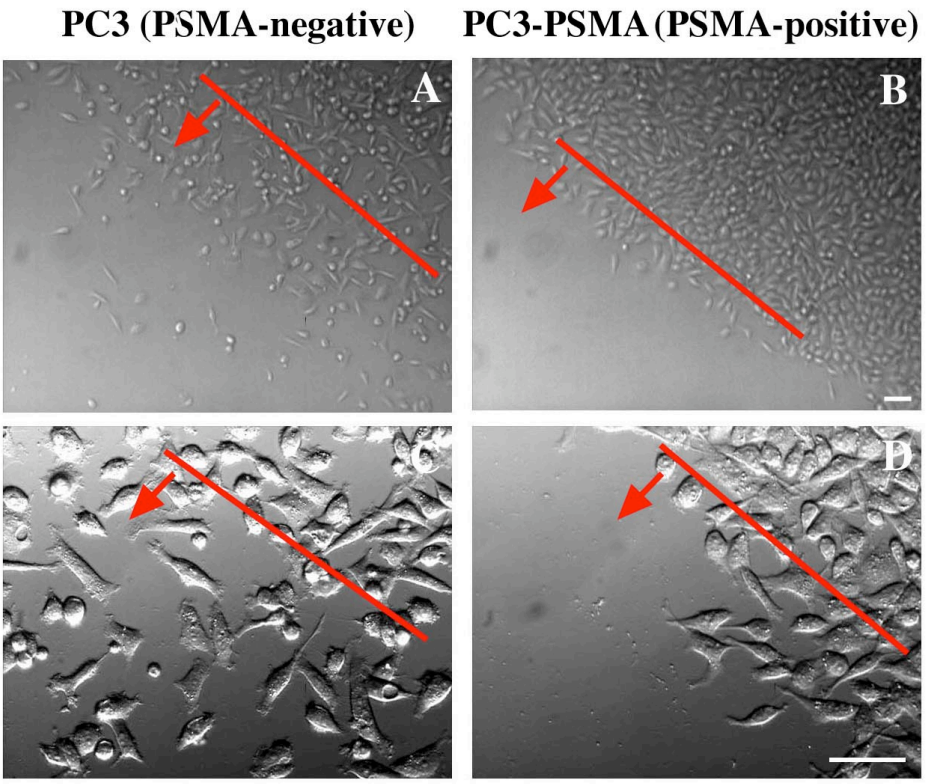

Figure 3. Expression of PSMA in PC3 cells reduces its cell motility.
PC3 and PC3 cells stably transfected with PSMA (PC3-PSMA) were grown to confluent monolayer before wounding. Cells were recovered for 1 hour after wounding in regular growth medium and DIC images were collected after 6 hrs in growth medium (A-D). Images in C and D are higher (3X) magnification comparing to images in A and B. The lines indicate the edges of wounded area and the arrows point to the direction of cell migration. Bar=20uM.

Result: Expression of PSMA in PC3 cells slows cell migration.

Figure 4. Cell Motility of PC3 and PC3-PSMA Cells

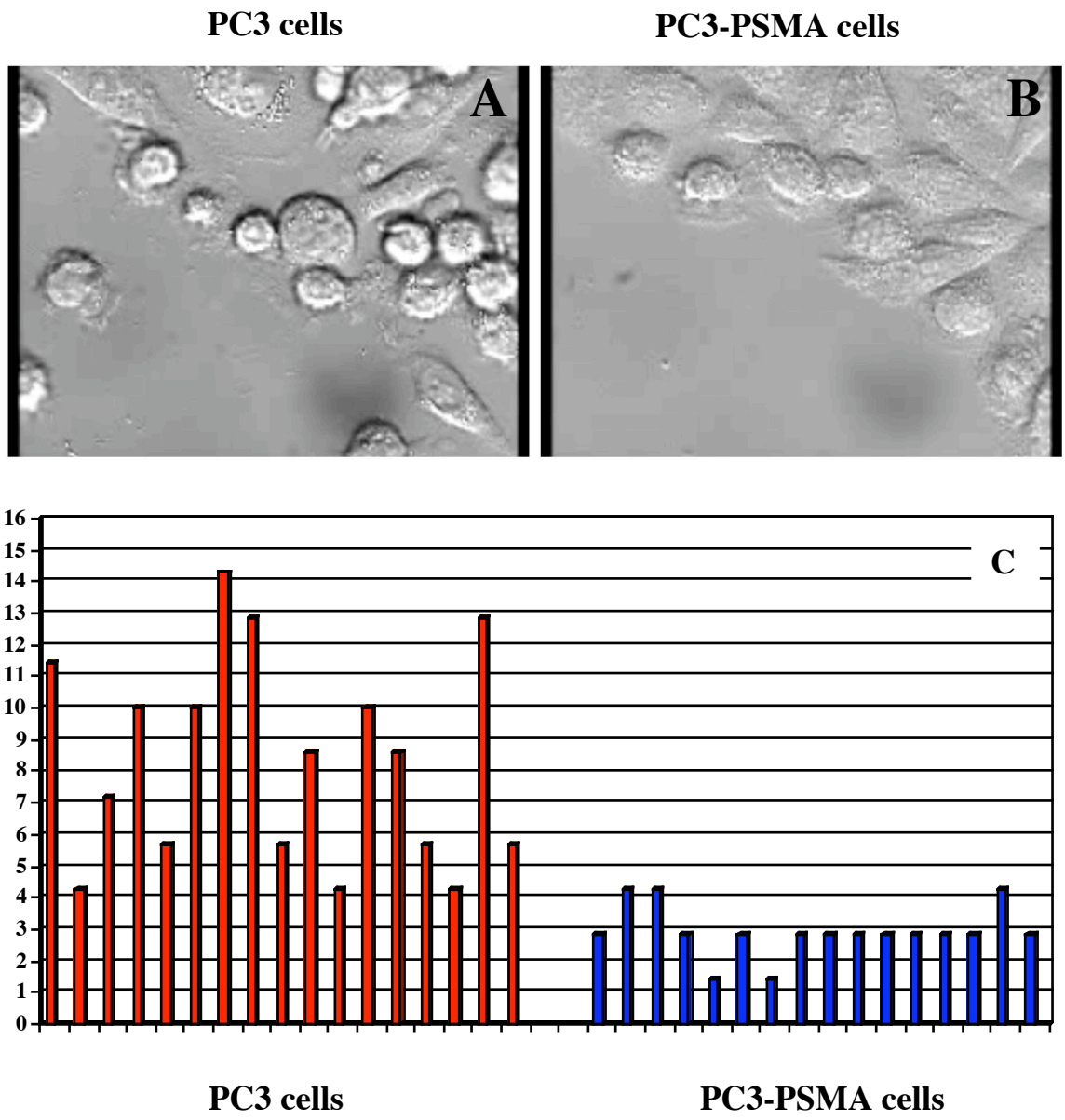

Figure 4. Live cell motility of PC3 and PC3-PSMA cells
Live cell motility of PC3 (A) and PC3-PSMA (B) cells was recorded after wounding. Total recording time=30min. In C, the distances of cell migration of PC3 (n=17) and PC3-PSMA (n=16) shown in Fig. 4A-B were measured on the migratory tracks produced by individual PC3 and PC3-PSMA cells shown in A-B. The X-axis indicates the individual cells and the Y-axis showes the values of the relative distances the cell migrated during the recorded time.

Result: PSMA expression reduces PC3 cell motility.

Figure 5. Effect of Reduced PSMA Expression on LNCaP Cell Motility

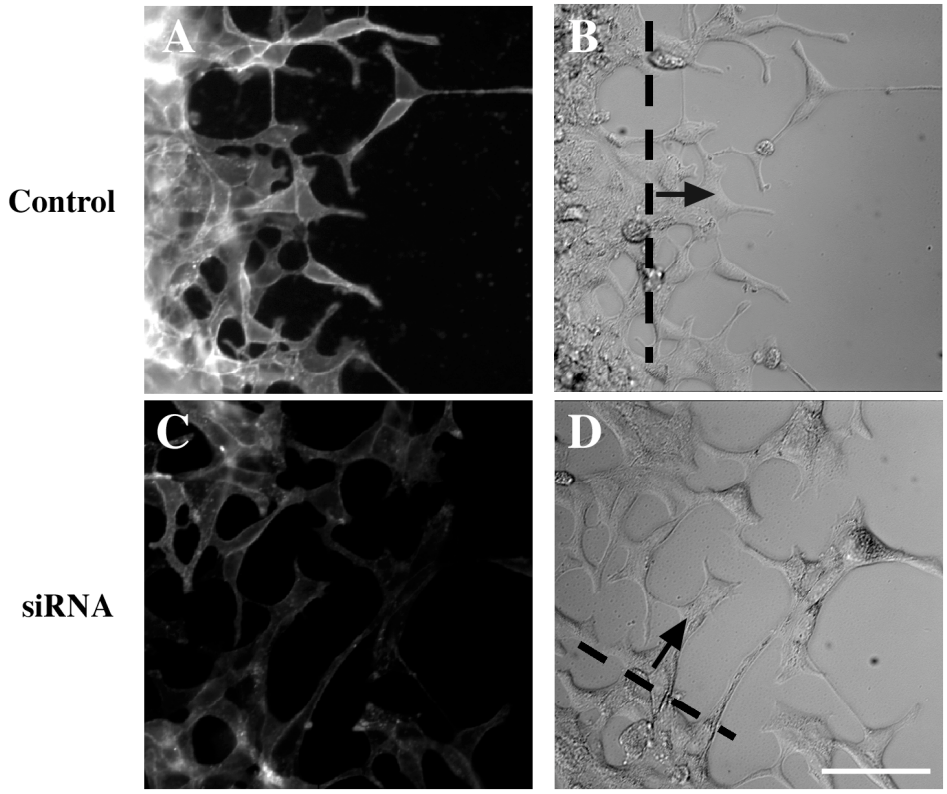

Figure 5. Reduction of PSMA expression increases PSMA-positive LNCaP cell motility. Control LNCaP cells (A-B) and LNCaP cells transfected with siRNA-PSMA (C-D) were grown until confluence before wounding. After wounding, cells were recovered for 1 hour after wounding in regular growth medium and were grown in medium without serum for 48 hr. DIC images were collected after 48 hrs in growth medium without serum (A-D). The dotted lines indicate the edges of wounded area and the arrows point to the direction of cell migration. Bar=20uM.

Result: Reduction of PSMA expression by siRNA increase LNCaP cell motility. Significantly higher numbers of cells with low PSMA expression (C-D) was migrated into the wounded area. Interestingly, these transfected cells (C-D) also migrated for longer distance when compared with the control untransfected LNCaP cells that had higher PSMA levels (A-B)

Figure 6. Distribution of F-actin in 22Rv1 Cells with Different PSMA Expression Levels

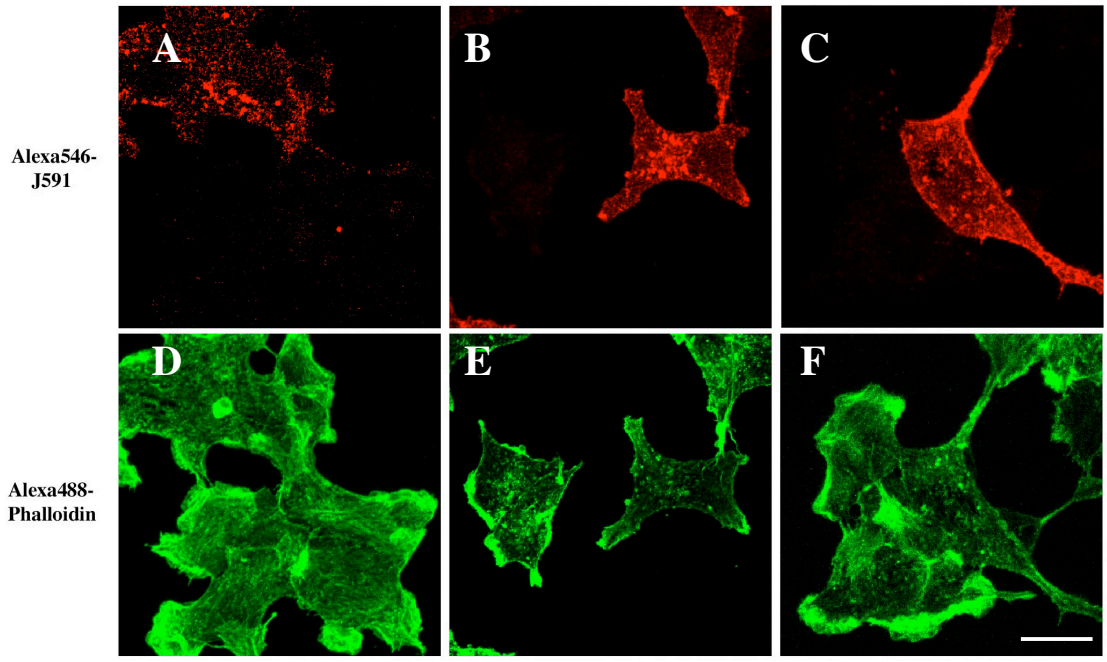

Figure 6. Distribution of PSMA and F-actin in 22Rv1 cell
22Rv1 cells fixed in 3.3% paraformaldehyde and permeabilized with 0.025% saponin were stained with Alex546-J591 (A-C) and Alexa488-phalloidin (D-F). Projections of confocal images from three different fields (A&D, B& E and C&F, respectively) of the same experiment are shown. Bar=10um.

Results: Different levels of PSMA expression were observed in 22Rv1 cells and alteration in F-actin organization was observed in 22Rv1 cells with higher levels of PSMA expression**.**